REMARKS/ARGUMENTS

The Pending Claims

Claims 1 and 4-18 are pending and directed to a method for the characterization of primary tumors or separate areas of primary tumors.

Amendments to the Specification

The specification has been amended to capitalize the trademarked names POLYMORPHPREP™ and NYCOPREP™ and to recite the components, as supported by claim 13, as originally filed. Accordingly, no new matter has been added by way of these amendments.

Amendments to the Claims

The claims have been amended to point out more particularly and claim more distinctly the invention. In particular, claim 1 has been amended to recite the method comprises isolating or concentrating clusters of tumor cells contained in a sample material, determining the genotype of the polymorphic DNA sequences of microsatellite markers of the clusters of tumor cells, and characterizing the primary tumor or separate areas of the primary tumor according to the genotype of polymorphic DNA sequences. This amendment is supported by claims 2 and 3 (now canceled) and the application at, for example, paragraphs [0014]-[0016] and [0022] of U.S. Patent Application Publication 2006/0147911 ("the '911 publication").

Claim 8 has been amended to insert sequence identifiers after each sequence. Claims 1, 6, and 9-16 have been amended to clarify the claim language.

Claims 17 and 18 are new and are supported by original claims 6 and 8.

No new matter has been added by way of these amendments.

Summary of the Office Action

The Office requests that the trademarked terms POLYMORPHPREP™ and NYCOPREP™ be capitalized and accompanied by generic terminology when used in the specification. The Office objects to claim 8.

The Office rejects claims 1-16 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. The Office rejects claims 1-16 under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement.

The Office rejects claims 1-3, 5, 15, and 16 under 35 U.S.C. § 102(b) as allegedly anticipated by Sidransky et al. (WO 96/06951).

The Office rejects claims 4, 6, and 7 under 35 U.S.C. § 103(a) as allegedly unpatentable over Sidransky et al., Jenkins et al. (*Genes, Chromosomes, and Cancer, 21*: 131 (1998)), and Osman et al. (*Intl. J. Cancer, 71*: 580 (1997)). The Office rejects claim 9 under 35 U.S.C. § 103(a) as allegedly unpatentable over Sidransky et al. and Zhang (*Nucleic Acids Research, 27*: e36 (1999)). The Office rejects claims 10-13 under 35 U.S.C. § 103(a) as allegedly unpatentable over Sidransky et al. and Brandt et al. (*Clinical Chemistry, 42*: 1881 (1996)). The Office also rejects claim 14 under 35 U.S.C. § 103(a) as allegedly unpatentable over Sidransky et al. in view of Girard et al. (*Cancer Research, 60*: 4894 (2000)).

Reconsideration of these rejections is hereby requested.

Discussion of the Use of Trademarked Terms in the Specification

As requested by the Office, the specification has been amended to capitalize the trademarked names POLYMORPHPREP and NYCOPREP and list generic terminology.

Discussion of the Claim Objection

The Office contends that claim 8 contains sequences without listing sequence identifiers. Claim 8 has been amended to recite sequence identifiers for the particular sequences. Accordingly, Applicants request the withdrawal of the claim objection.

Discussion of the Indefiniteness Rejections

The Office contends that claim 1 is unclear because it lacks a positive process step relating back to the preamble. Claim 1, as amended, recites several method steps that include isolating or concentrating clusters of tumor cells contained in a sample material, determining the genotype of polymorphic DNA sequences of microsatellite markers of the isolated or concentrated clusters of tumor cells contained in the sample material, and characterizing the primary tumor or separate areas of the primary tumor according to the genotype of polymorphic DNA sequences.

The Office contends that claim 3 is unclear due to the phrase "alterations therein." Claim 3 has been canceled.

The Office contends that claim 5 is unclear because there is insufficient antecedent basis for "the polymorphic DNA." Claim 1, as amended, recites polymorphic DNA. Claim 5 depends from claim 1. Accordingly, there is sufficient antecedent basis for "the polymorphic DNA" in claim 5.

The Office contends that claim 10 is unclear because there is insufficient antecedent basis for "the isolation and concentration of tumor cells cytokeratin-positive cells." Claim 10, as amended, recites that the tumor cells isolated from the sample material are cytokeratin-positive cells and/or epithelial cells positive for tissue-specific proteins.

The Office contends that claim 11 is unclear due to the phrase "if necessary after homogenization in a solvent." Claim 11, as amended, no longer recites this phrase.

The Office contends that claim 12 is unclear because there is insufficient antecedent basis for "the medium." Claim 12, as amended, refers to a hyper-osmotic medium.

For the above-described reasons, the claims are sufficiently clear. Applicants request that the indefiniteness rejections be withdrawn.

Discussion of the Enablement Rejections

The Office contends that the claims are not enabled for detection of any tumor using any cell cluster isolated by any isolation method comprising analysis of any genetic change in any amplification method. Applicants traverse this rejection for the following reasons.

The pending claims, as amended, are directed to a method for the characterization of primary tumors or separate areas of primary tumors comprising isolating or concentrating clusters of tumor cells contained in a sample material, determining the genotype of the polymorphic DNA sequences of microsatellite markers of the clusters of tumor cells, and characterizing the primary tumor or separate areas of the primary tumor according to the genotype of polymorphic DNA sequences.

In the inventive method, cell clusters or clustered cells isolated from body fluids, rather than isolated tumor cells, can be used to characterize solid primary tumors. Based on the description in the specification (see, e.g., paragraphs [0022]-[0202] of the '911 publication) and what was known in the art at the effective filing date of the application, one of ordinary skill in the art would recognize that the cell clusters can be isolated from a liquid sample by many means, e.g., by weight. One of ordinary skill in the art would have understood how to separate cell clusters from single cells.

The specification also describes the isolation of the clusters from tumor cells from several different samples, including blood, urine, and fluid from nipple aspiration of the female breast (see, e.g., paragraphs [0015], [0051]-[0192], and [0244]-[0247] of the '911 publication). Additionally, the specification describes how the inventive method can be used for the characterization of several different primary tumors, such as breast carcinoma, ovary carcinoma, prostate carcinoma, colon carcinoma, stomach carcinoma, and bladder carcinoma (see, e.g., paragraphs [0002], [0051]-[0192], and [0244]-[0247] of the '911 publication).

Furthermore, the specification discloses that the connection between the formation and spread of malignant tumors and an accumulation of multiple genetic changes is well-known, which changes can be identified using microsatellite markers (see, e.g., paragraph [0013] of the '911 publication. For example, Ankar et al. (*Cancer and Metastasis Reviews, 18*: 65-73 (1999); copy enclosed) discloses the use of microsatellite markers for the

characterization of primary tumors. Thus, one of ordinary skill in the art would recognize that the polymorphic DNA at microsatellite markers can be used to determine tumor development, metastasizing potential, therapy requirements, efficacy of therapy, and assessment of the course of a disease or therapy as set forth in the specification at, for example, paragraphs [0001] and [0212]-[0247] of the '911 publication.

Accordingly, based on the description in the specification and what was known in the art at the effective filing date of the application, one of ordinary skill in the art would have recognized how to use the inventive method for the reliable staging and prognosis of tumors.

Discussion of the Anticipation Rejection

The Office contends that Sidransky et al. anticipates the subject matter of claims 1-3, 5, and 15-16.

As set forth above, the pending claims, as amended, are directed to a method for the characterization of primary tumors or separate areas of primary tumors comprising isolating or concentrating clusters of tumor cells contained in a sample material, wherein the sample material is selected from the group consisting of blood, urine, and nipple aspiration fluid from the female breast; determining the genotype of the polymorphic DNA sequences of microsatellite markers of the clusters of tumor cells; and characterizing the primary tumor or separate areas of the primary tumor according to the genotype of polymorphic DNA sequences.

Sidransky et al. does not teach or suggest that the cells of *cell clusters* can be analyzed to characterize primary tumors. In particular, Sidransky et al. does not teach or suggest isolating clusters of tumor cells from body fluids (e.g., blood, urine, and nipple aspiration fluid from the female breast) to characterize solid tumors, as required by the pending claims.

Since Sidransky et al. does not teach or suggest every element of the pending claims, Sidransky et al. cannot be considered to anticipate the pending claims. Accordingly, Applicants request that the anticipation rejection is withdrawn.

Discussion of the Obviousness Rejections

In order to establish a *prima facie* case of obviousness with respect to a claim, at least two criteria must be met: (1) the prior art references must suggest to one of ordinary skill in the art to make the subject matter defined by the claims in issue and (2) the prior art references must provide one of ordinary skill in the art with a reasonable expectation of success in so making the subject matter defined by the claims in issue. Both the suggestion and the reasonable expectation of success must be found in the prior art references, not in the disclosure of the patent application in issue. The prior art references must teach or suggest all the claim limitations. See, e.g., *In re Vaeck*, 947 F.2d 488, 493, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991).

As set forth above, Sidransky et al. does not teach or suggest every element of the pending claims. Furthermore, the Jenkins, Osman, Zhang, and Girard references do not remedy the deficiencies of Sidransky et al. For example, the Jenkins, Osman, Zhang, and Girard references do not teach or suggest isolating clusters of tumor cells from body fluids (e.g., blood, urine, and nipple aspiration fluid from the female breast) to characterize solid tumors, as required by the pending claims.

Therefore, since the Sidransky, Jenkins, Osman, Zhang, and Girard references, either alone or in combination, do not provide all of the elements of the pending claims, these references cannot be considered to render obvious the pending claims.

One of ordinary skill in the art would not have been motivated to prepare cancer cell clusters by the method described in Brandt et al. for use in the assay of microsatellite loci to detect primary tumors of Sidransky et al, as set forth by the Office. Brandt et al. discloses the preparation of cancer cell clusters by density gradient separation, but does not disclose that primary tumors or separate areas of primary tumors can be characterized by determining the genotype of the polymorphic DNA sequences of microsatellite markers of the clusters of tumor cells, as required by the pending claims. Since Sidransky et al. does not teach or suggest using clusters of tumor cells in the assay of microsatellite loci to detect tumors, one of ordinary skill in the art would not have recognized that the tumor cells of Sidransky et al. could successfully be replaced with clusters of tumor cells. Moreover, even if one of ordinary skill in the art were motivated to combine Sidransky et al. and Brandt et al., one

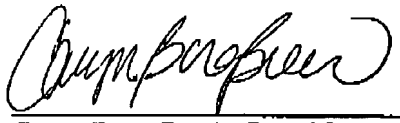
would not necessarily combine the cited references in the precise manner necessary to arrive at the inventive method.

For the above-identified reasons, the inventive method cannot be considered to be obvious in view of the cited references. Therefore, Applicants request that the obviousness rejections are withdrawn.

Conclusion

Applicants respectfully submit that the patent application is in condition for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



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Date: June 6, 2007

Cancer and Metastasis Reviews **18:** 65–73, 1999.

Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients

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Key words: mutation detection, microsatellite DNA, circulating DNA, cancer, plasma/serum

Abstract

Small amounts of free DNA circulate in both healthy and diseased human plasma/serum, and increased concentrations of DNA are present in the plasma of cancer patients. Characteristics of tumour DNA have been found in genetic material extracted from the plasma of cancer patients. These features include decreased strand stability and the presence of specific oncogene, tumour suppressor gene and microsatellite alterations. Point mutations of the ras genes have been detected in the plasma DNA of patients suffering from haematopoetic malignancies, colorectal and pancreatic cancer, sometimes prior to clinical diagnosis. Rearranged immunoglobulin heavy chain DNA has been found in the plasma of patients with non-Hodgkins lymphoma and acute B cell leukaemia. Microsatellite instability, expressed either as a new allele or a loss of one allele (LOH) occurs in the plasma and serum DNA of patients suffering from head and neck, lung and renal cell cancer. The results obtained in many different cancers have opened a new research area indicating that plasma DNA might eventually be a suitable target for the development of non-invasive diagnostic, prognostic and follow-up tests for cancer.

Over 50 years ago, and 5 years before Watson and Crick elucidated the double-helical structure of DNA, Mandel and Métais identified nucleic acids in human blood plasma [1]. Using a perchloric acid precipitation method, they found RNA and DNA in the plasma of both normal subjects and those with various diseases, the mean quantity of DNA being approximately 1 mg/l. Following this early work, the subject of plasma and serum DNA appears to have been largely forgotten, and it was not until the 1960s that interest in circulating DNA reawakened with the discovery of DNA in the serum of patients with Systemic Lupus Erythematosus (SLE) [2]. Further work showed that DNA was not confined to the plasma of patients with SLE, but was also found in patients with other diseases including rheumatoid arthritis, glomerulonephritis, pancreatitis, cholelithiasis, inflammatory bowel disease, peptic ulcer disease, hepatitis and oesophagitis [3–6]. Increasingly sensitive assays were also able to detect DNA in the serum and plasma of normal healthy subjects, usually at levels between 10 and 30 ng/ml plasma [4].

DNA is present in increased amounts in plasma/serum DNA of cancer patients

Leon and colleagues [7] found that cancer patients tended to have even higher levels of circulating DNA than those with non-malignant diseases. The level of free DNA in the serum of 173 patients with various types of cancer, and in 55 healthy individuals, was determined by a radioimmunoassay where the serum of a Lupus patient served as the source of antibody. DNA concentration in the normal controls had a mean of 13 ng/ml while in the cancer patients the mean was 180 ng/ml [8]. Although no correlation was found between circulating DNA levels and the size or location of the primary tumour, significantly higher DNA levels were found in the serum of patients with metastases

compared to localised disease. Furthermore, DNA levels decreased by up to 90% following radiotherapy, especially in lymphoma, lung, ovary, uterus and cervical tumours. In contrast, persistently high or increasing DNA levels were associated with a lack of response to treatment. This conclusion was confirmed later on a study of patients with benign or malignant gastrointestinal disease. Patients with benign disease had mean DNA levels of 118 ng/ml compared with 412 ng/ml for those with a variety of neoplastic diseases [9].

Working with various malignancies (leukaemia, lymphoma, lung, breast and gastrointestinal tumours) and after having extracted and purified the DNA from the plasma, it was found that detectable amounts of circulating DNA were found only in patients with advanced malignancies bearing a large tumour cell burden and that in two cases with progressive cancer where a second determination could be performed in the course of the disease, an increased plasma DNA concentration was measured suggesting a relation between this parameter and tumour evolution. The purified DNA was shown to be composed of double-stranded fragments ranging up to 21 kb in length and, using a ^{32}P-labelled human DNA probe, identified as human in origin [10].

Further work by researchers working with lung cancer patients found a correlation between circulating levels of DNA and prognosis [11,12]. In these studies plasma DNA levels were increased in cancer patients as compared to normal subjects, were higher in patients with advanced disease and correlated with other tumour markers including serum lactate dehydrogenase activity and neurone-specific enolase concentrations. In addition, survival was poor in patients with plasma DNA greater than 100 ng/ml [12].

Tumour DNA in the plasma/serum of cancer patients: Biophysical properties

In the results showing increased levels of plasma DNA in cancer patients it was not determined if the circulating DNA was released from activated lymphocytes reacting towards the disease as some data from SLE studies might suggest [5] or from the tumour cells themselves. One way to solve the problem was to take advantage of differences between properties of the DNA of malignant and normal cells, and to find out if the DNA isolated from the plasma of cancer patients presented some of the characteristics detected in the DNA of malignant cells. It is known that cancer cell DNA have a biophysical characteristic: decreased strand stability [13]. Due to this characteristic, upon exposure to certain chemical carcinogens, DNA strand separation can be measured by the increase in ultraviolet absorbance (hyperchromicity) which, in the case of cancer cell DNA, appears at a lower temperature. Moreover, these same carcinogens at given concentrations stimulate *in vitro* DNA synthesis in a nucleoside triphosphate DNA-polymerase-containing system much more strongly in the presence of a DNA template obtained from neoplastic cells than from normal cells. The plasmatic DNA samples from cancer patients presented an increased *in vitro* synthesis ranging from 65% to 300%. In contrast, no increase was observed with control DNA of healthy donors. Moreover, all plasma DNA from cancer patients presented a hyperchromic effect at room temperature when carcinogens were added. Control DNAs presented a hyperchromic effect only in presence of KOH. These experiments showed clearly that an important part of the DNA found in the plasma of cancer patients originated from the tumour [14].

Tumour DNA in the plasma/serum of cancer patients: Ras mutations and rearranged Ig heavy chain

Further research to define the nature of circulating DNA was aided by the development of the polymerase chain reaction (PCR) in the late 1980s. The first mutational analyses performed on plasma DNA concentrated on the K-ras and N-ras oncogenes [15–17], known to be mutated in a variety of cancer types [18–21]. Indeed, over 90% of pancreatic carcinomas [18] and approximately 50% of colorectal carcinomas [19] harbour a point mutation of the K-ras gene. In addition, the N-ras gene is mutated in approximately 40% of patients with acute myeloblastic leukaemia or myelodysplastic syndrome [20,21].

Plasma DNA alterations and colorectal cancer

To date, four studies have examined the association between colorectal cancer and the presence of mutations in plasma DNA (Table 1). All four studies

Table 1. Summary of plasma DNA studies in cancer patients

Year/reference	Tumour type	Number of subjects	Gene(s) studied	Plasma DNA mutations (%)
1994 [17]	MDS/AML	10	N-ras	50
1994 [15]	Pancreas	3	K-ras	100
1996 [33]	Head and neck	21	MA	29
1996 [36]	SCLC	21	MA	71
1997 [22]	Colorectal	14	K-ras	43
1997 [23]	Colorectal	14	K-ras	43
1997 [24]	Colorectal	31	K-ras	39
1997 [29]	Lymphoma	110	Ig Heavy chain	86
1998 [25]	Pancreas	21	K-ras	81
1998 [26]	Pancreas	21	K-ras	43
1998 [38]	Renal cell	40	MA	63
1998 [39]	Colorectal	44	K-ras, p53, MA	23
1998 [37]	NSCLC	22	MA	28
1999 [30]	NSCLC	22	Hypermethyl.	73
1999 [31]	liver	22	Hypermethyl.	73
1999 [40]	Colorectal	17	p53	29
1999 [41]	SCLC	10	p53	10
	breast	15	p53	13

MA: microsatellite alternations.

have used assays to detect K-ras mutations, primarily because relatively simple and reliable PCR techniques exist to detect such alterations. An initial study of the K-ras gene, performed by Vasioukhin et al. [16] on 14 patients using sequence specific primers, identified mutations in the primary tumours of 7 patients. Identical mutations were found in the plasma of 6 of these (86%), but no plasma DNA alterations were detected either in colorectal cancer patients without K-ras mutations within the primary tumour or in healthy controls. Further work, using both a restriction fragment length polymorphism PCR (RFLP) assay, sequencing and cloning techniques confirmed these results [22]. Of interest is that mutations were not only found in the plasma of patients with advanced disease, but also in circulating DNA extracted from patients with stage I and II disease. These initial results have since been confirmed [23].

In another work plasma or serum was fractionated from the blood of 31 patients with metastatic or unresected colorectal cancer and from 28 normal volunteers. DNA was amplified in a two-stage PCR assay using selective restriction enzyme digestion to enrich for mutant K-ras DNA. Mutant K-ras DNA was detected in the plasma or serum of 12 (39%) patients, all confirmed by sequencing, but was not detected in any of the controls. K-ras mutations were detected in plasma or serum regardless of primary tumour location, principal site of metastasis or proximity of chemotherapy and surgery to blood sampling. Tumour specimens showed the same mutations as the blood specimens [24].

Plasma DNA K-ras mutations in pancreatic cancer

Pancreatic cancer differs from colorectal cancer in a number of important respects when it comes to detecting gene alterations in plasma. Firstly, pancreatic cancer can be difficult to differentiate from other conditions on the basis of clinical features and imaging investigations, so that a simple and non-invasive test would be especially valuable in this condition. Secondly, the K-ras gene is mutated in approximately 90% of pancreatic adenocarcinomas [18], suggesting that a comprehensive analysis of many genes would be unnecessary to detect the majority of cases. Three studies (Table 1) have therefore concentrated on detecting this gene in pancreatic cancer.

Using allele-specific primers and RFLP-PCR followed by sequencing, Sorenson et al. found plasma DNA K-ras alterations in three patients [15], and mutations were identical in tumour and plasma. In another study [22], plasma DNA was isolated from 21 pancreatic cancer patients and K-ras alterations detected by RFLP-PCR assay and subsequent product sequencing. Patients were followed up to determine their clinical outcome. K-ras mutations was found in the plasma of 17 patients (81%). In cases in which both plasma and pancreatic tissue were available, DNA mutations

were similar in corresponding plasma and tissue samples. Plasma DNA alterations were found 5–14 months before clinical diagnosis in 4 patients. Mutant DNA was not found in the plasma of 2 patients with chronic pancreatitis or in 5 healthy controls.

A third study looking at pancreatic adenocarcinoma also resulted in plasma DNA alterations being detected in a high proportion of cases in which a K-ras mutation was found in tumour tissue (26). In this study, patients with plasma DNA mutations tended to have larger tumours and were less likely to have a potentially curative procedure. In addition, treatment resulted in disappearance of K-ras gene mutations from plasma DNA in 6 of 9 patients (67%) which had a follow-up, while the three patients with persistently positive plasma specimens had either an early recurrence or progressive disease. Other features, including patient age or sex, histological type, mode of invasion and metastasis did not correlate with the presence of mutated plasma DNA.

Some recent studies have demonstrated K-ras mutations in microdissected tissues taken from patients with pancreatic hyperplasia either in the presence or absence of chronic pancreatitis [27,28]. However, an interesting feature of plasma DNA studies is that K-ras mutations were not found in patients with chronic pancreatitis [25,26]. In view of the fact that the presence of mutant plasma DNA is related to tumour size [26], it is unlikely that patients with pancreatic hyperplasia would manifest plasma DNA mutations, but further work using even more sensitive assays is required to determine this more clearly.

Plasma DNA N-ras mutations and rearranged Ig heavy chain DNA in haematological malignancies

N-ras mutations have been found in DNA extracted from the bone marrow of patients with myelodysplastic syndrome and acute myelogenous leukaemia (AML), and these alterations have been examined in the plasma, leucocytes and bone marrow of such patients [17]. In patients with N-ras alterations, mutant DNA was always present in plasma DNA, though sometimes absent in the DNA of peripheral blood cells or bone marrow. This indicates that a single bone marrow biopsy or aspiration does not necessarily contain all the malignant clones involved in the disease and that plasma might be a suitable template for detecting alterations in patients suspected of having myelodysplastic syndrome or AML.

Rearranged Ig heavy chain DNA has also been detected in cell-free blood samples of patients with B cell malignancies [29]. When examined prior to treatment clonotypic DNA was found in serum or plasma in 86% of 110 patients with non-Hodgkin's lymphoma and acute B-precursor lymphoblastic leukaemia. Follow-up showed a close correlation between persisting tumour-derived plasma/serum DNA and resistant disease or early relapse.

Aberrant hypermethylation of tumor suppressor genes in plasma/serum DNA of lung and liver cancer

Recent evidence shows aberrant DNA methylation can also be found in the serum of cancer patients. Twenty-two patients with non-small-cell lung cancer were tested using methylation-specific PCR, searching for promoter hypermethylation of the tumour suppressor gene p16, the putative metastasis suppressor gene death-associated protein kinase, the detoxification gene glutathione S-transferase P1, and the DNA repair gene O6-methylguanine-DNA-methyltranferase. Aberrant methylation of at least one of these genes was detected in 15 of 22 (68%) NSCLC tumours but not in any paired normal lung tissue. In these primary tumours with methylation, 11 of 15 (73%) samples also had abnormal methylated DNA in the matched serum samples [30]. Similarly, in the study of liver cancer, p16 methylation was found in 73% (16/22) of hepatocellular carcinoma tissues. Amongst the 16 cases with aberrant methylation in the tumour, similar changes were also detected in the plasma/serum samples of 81% (13/16) of the cases. No methylated p16 sequences were detected in 38 patients with chronic hepatitis cirrhosis or in 10 healthy control subjects [31].

Microsatellite alterations in plasma/serum of head and neck, lung and renal cell cancer patients

Microsatellite DNA alterations, defined as the presence of new alleles (shifts) or loss of heterozygosity (LOH), are an integral part of neoplastic progession and valuable as clonal markers for human cancer [32]. Such alterations have also been detected in the plasma of patients with various cancers.

Using 12 microsatellite markers, a study on paired plasma and lymphocyte samples from head and neck

cancer patients detected microsatellite alterations in the plasma of 6 of 21 patients (29%) matching those found in tumour tissue [33]. All 6 patients had advanced (stage III or IV) disease with a correspondingly poor outcome. A more recent study on 102 patients with head and neck cancer, but using only two markers, also demonstrated the presence of mutant DNA in the plasma of 19% of patients with positive tumours [34].

Microsatellite alterations are also frequently associated with lung cancer. Using a set of three primer pairs, Sidransky's group have found one trinucleotide repeat sequence and two tetranucleotide repeat sequences, to be altered altogether in 50% of SCLC tumours [35]. The same alterations were found in the sputum [32,35]. Therefore, it seemed sensible to search for microsatellite alterations in the plasma of such cases. Using the same markers on tumour tissue, lymphocytes and plasma DNA, microsatellite alterations were detected in 16 of 21 small-cell lung cancers, and identical mutations were present in the plasma DNA of 15 of these cases (94%) [36]. Follow-up plasma DNA testing of patients with incurable disease identified the same microsatellite pattern as that found in original plasma specimens. Further study showed that microsatellite alterations were not limited to the plasma DNA of patients with small-cell lung cancers, but could also be detected in DNA extracted from the serum of non-small-cell lung cancer patients [37]. Using 4 microsatellite markers directed at chromosome 3p, Sanchez-Cespedes et al. found alterations in the serum of 6 of 22 patients (28%) undergoing curative surgery, including one patient with early (stage I) disease.

Finally, chromosome 3p deletions in plasma DNA have also been studied in patients with clear cell renal carcinoma [38]. Using a panel of 5 highly polymorphic microsatellite markers corresponding to the Von Hippel–Lindau tumour suppressor gene, LOH was found in at least one locus in 63% of the plasma samples (25/40), while a shift was found in one patient. As with lung cancer patients, no association with tumour stage was apparent.

Limitations of microsatellite analysis in plasma

The use of microsatellite markers in plasma/serum DNA of cancer patients presents however limitations as LOH or microsatellite alterations may be masked by normal DNA from the lymphocytes. There are no enrichment techniques as is the case for oncogene or tumour suppressor gene mutations. For instance, although LOH or shifts were found in 80% of primary tumour (35/44) of colorectal cancer patients, no alterations were detected in paired serum DNA. This does not mean that there is no tumour DNA present in the serum since ras mutations and p53 mutations could be found [39]. Taken together, either a K-ras or a p53 mutation was detected in the serum in 40% of the 25 patients whose primary tumour contained a mutation. Five of seven stage B patients with a p53 mutation in the tumour presented a mutation in the paired serum.

This work indicates that the use of microsatellite instability in the plasma as tumour markers greatly depends on the kind of cancer studied. If it seems possible to envisage with this approach in the future a detection test for some malignancies, for others, only a prognosis test or a non-invasive way to follow-up seems possible. In the case of some malignancies, oncogene or tumour suppressor gene mutations will have to be used. Recently, p53 mutations have also been found in the plasma DNA of large bowel carcinoma [40], breast and lung cancer [41].

In any case, it would be very interesting to compare the sensitivity of plasma/serum DNA analysis and other methods such as micrometastase detection in the bone marrow [42].

The origin and mechanism of release of DNA into plasma

Although it is evident that DNA circulates freely in blood plasma both in disease and in health, the source of this DNA remains enigmatic. It can be presumed that circulating DNA in healthy subjects derives from lymphocytes or other nucleated cells. Yet, it is not known why cancer patients have such large quantities of plasma DNA, nor where this genetic material derives from. As with normal subjects, a proportion seems to originate from lymphocytes, since wild-type DNA has been detected in the plasma of all cancer patients studied so far. However, a substantial proportion of plasma DNA in cancer patients derive from tumour cells. This latter concept is supported by both quantitative and qualitative observations. Plasma DNA levels are not only greater in cancer patients than in normal subjects, but also correlate inversely with outcome and tend to fall with effective treatment [8,12]. In addition, the ability to detect LOH in plasma DNA [33,36–38], suggests that mutant DNA is the predominant subtype in at least some cancer patients.

The most common hypothesis advanced for circulating DNA in the plasma of cancer patients is that it is due to the lysis of circulating cancer cells or micrometastases shed by the tumour. This is clearly not the case since there are not enough circulating cells to justify the amount of DNA found in the plasma. Sorenson [43] calculated that in relation to the amount of DNA he found in the plasma of pancreatic cancer patients there would have to be one thousand cancer cells per ml which is far more than has ever been found. Using another DNA extraction procedure (Boehringer columns) which yields ten times more DNA than the method used by Sorenson (Qiagen Kit) it would have to be assumed that ten thousand tumour cells per ml are circulating in the blood stream. Now, in the case of colorectal patients, no ras mutations have been found in the cells of the Ficoll layer where micrometastatic cells should be found. Moreover, the same mononuclear cells which should include circulating tumor cells if there are any were used as control of normality in all microsatellite analysis [33–38].

It thus appears that tumour DNA shed in the blood stream could be due either to DNA leakage resulting from tumour necrosis or apoptosis or to a new mechanism of active release.

Tumour necrosis has been postulated since higher amounts of DNA were found in plasma of patients with large tumours or with advanced diseases with metastases [9–12,33], although it should be reminded that plasma tumour DNA is also found in early stages [16,22,25,37,38]. An argument against necrosis is that after radiation therapy, the plasma DNA levels decreased up to 90% [8], whereas one might expect an initial plasma DNA surge following radiotherapy if necrosis was the predominant pathway for DNA release.

Apoptosis has been advanced as the origin of circulating DNA on the basis of several observations, the main one being that plasma or serum DNA often presents ladder pattern after electrophoresis which reminds of the pattern shown by apoptotic cells [10,12,44]. Giacona et al. [44] have evaluated the plasma DNA by gel electrophoresis and measured the variation in length of soluble DNA fragments by electron microscopy in plasma from three patients with pancreatic cancer and from 3 healthy controls. Nick-translated DNA isolated from plasma and subjected to gel electrophoresis and electron microscopy displays different band patterns depending on whether the plasma originates from a normal subject or cancer patient. Normal plasma DNA exhibits autoradiographic bands at sizes equivalent to whole-number multiples (1–5×) of nucleosomal DNA (185–200 bp). In contrast, plasma DNA from pancreatic cancer patients displays a stronger ladder-like pattern. Furthermore, strand length distributions of DNA (DNA-SL) in the two groups tend to differ, with a plasma DNA-SL of 311 nm compared to 231 nm for control and cancer patients respectively. Finally, small excesses of DNA at approximately 63, 126, 189, 252 and 315 nm are more prominent in the plasma of cancer patients. The authors conclude that a significant proportion of plasma DNA derives from apoptosis of neoplastic cells. Using the same arguments, Fournié came to the same conclusions [12]. Let us not forget however that apoptosis is a mechanism lost by proliferating cancer cells.

As a third possibility it may be hypothesized that the tumour actively releases DNA into the blood stream. If one cannot affirm that the circulating tumour DNA proceeds of the same mechanism as observed *in vitro* where lymphocytes or whole organs spontaneously release DNA [45–49], it remains possible and plausible that both phenomena are related. It is worth reminding some data on this subject.

Cells or even whole organs in culture spontaneously release a nucleoprotein complex within a homeostatic system [45,46]. The newly synthesised DNA is preferentially released [45,46]. Both characteristics argue against apoptosis. After phytohemagglutinin activation lymphocytes selectively replicate several copies of a limited portion of their genome, copies which are then excreted into the culture medium [47–49]. A portion of the DNA released by PHA-stimulated human lymphoblasts becomes bound to the plasma membranes of the cells and is actively capped. Capping is accompanied by shape change, and caps are localised to uropods [49]. The marked difference between released DNA and cell DNA indicates that this extracellular DNA is not derived from dying cells. That such a mechanism might occur *in vivo* has been observed on mice in which a release of DNA in plasma has been observed after injection of bacterial lipopolysaccharides which has a mitogenic effect comparable to PHA [50]. We have observed that cancer cells in culture release more DNA than normal cells, for instance cells from leukaemic patients compared to lymphocytes from healthy donors (unpublished results). It is thus not surprising if the circulating DNA is due to an active release to find more plasma DNA in cancer patients than in healthy controls.

The presence of tumour DNA in the plasma is probably the result, in variable proportions, of the different mechanisms which produce leakage or excretion of DNA.

Conclusion

Medical implications of circulating DNA

What are the implications of plasma DNA for clinical medicine? Firstly, the possibility of a non-invasive plasma DNA test for cancer diagnosis is challenging. In addition, certain cancers, such as pancreatic adenocarcinoma, can often cause diagnostic difficulty, and a reliable plasma based PCR assay would obviate the need for multiple biopsies or diagnostic laparotomy in a proportion of cases. Secondly, the association between mutant plasma DNA and tumour stage, the presence of residual disease and outcome suggests that it might potentially serve as a prognostic marker in the absence of clinically detectable metastases [26,33]. Perhaps more importantly, follow-up tests might be valuable in assessing a patient's response to therapy and identify local or distant disease recurrence at an asymptomatic and early stage.

Although there are a number of potential applications for plasma-based DNA assays, their clinical value will ultimately depend on their ability to detect either new or recurrent tumours. It will almost certainly be necessary to analyse a broad panel of genes associated with each cancer type to increase the sensitivity of testing. In addition, research focusing on the association between precancerous and early disease with the appearance of mutant plasma DNA will also help define its role as a diagnostic and screening test for cancer.

Key unanswered questions

Another possible medical implication is the still hypothetical role of circulating tumour DNA in the formation of some metastases. DNA has been shown to spontaneously pass from procaryotic cells to eucaryotic cells [51–53] where it may be transcribed. The passage of DNA from eucaryote to eucaryote cells may also occur [54–56]. In the case of DNA transfer between bacteria and higher organism the case of crown gall, a plant cancer is particularly interesting. It was found a long time ago that the DNA spontaneously released by the bacteria *Agrobacterium tumefaciens* [57,58] which was later found to be a plasmid [59] induced the tumour. Now crown gall can give rise to metastases. Plant cells are held together by the network of their pectocellulosic walls which prevent the migration of transformed cells. Thus in the case of plants, metastatisation is probably due to circulating DNA. This might occur also in mammals as shown by the malignant transformation of donor cells sometimes observed after a bone marrow graft [60].

Finally, outside the field of oncology, plasma DNA may be used in the future for the prenatal detection of genetic diseases since fetal DNA has been detected in maternal plasma or serum allowing sex determination [61,62]. Moreover the presence of donor DNA in plasma of graft recipient opens new possibilities for post-graft follow-up [63].

Acknowledgements

The authors acknowledge generous funding by La Ligue Suisse contre le Cancer, The Susan Komen Foundation, The Stanley Thomas Foundation, The O.J. Isvet Fund, le Fonds National Suisse pour la Recherche Scientifique and the Special Trustees of St Bartholomew's Hospital.

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